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Haem Synthase and Cobalt Porphyrin Synthase in Various Micro-organisms

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1. The preparation of a crude extract of *Clostridium tetanomorphum* containing cobalt porphyrin synthase but little haem-synthase activity is described. 2. The properties of cobalt porphyrin synthase in the clostridial extracts is compared with the properties of a haem synthase present in crude extracts of the yeast *Torulopsis utilis*. 3. Cobalt porphyrin synthase in extracts of *C. tetanomorphum* inserts Co^{2+} ions into the following dicarboxylic porphyrins in descending order of rate of insertion: meso-, deutero- and proto-porphyrins. Esterification renders meso- and deutero-porphyrins inactive as substrates. Neither the tetracarboxylic (coproporphyrin III) nor the octacarboxylic (uroporphyrin III) compounds are converted into cobalt porphyrins by the extract, but the non-enzymic incorporation of Co^{2+} ions into these two porphyrins is rapid. These extracts are unable to insert Mn^{2+}, Zn^{2+}, Mg^{2+} or Cu^{2+} ions into mesoporphyrin. 4. Crude extracts of *T. utilis* readily insert both Co^{2+} and Fe^{2+} ions into deutero-, meso, and proto-porphyrins. Unlike the extracts of *C. tetanomorphum*, these preparations catalyse the insertion of Co^{2+} ions into deuteroporphyrin more rapidly than into mesoporphyrin. This parallels the formation of haems by the *T. utilis* extract. 5. Cobalt porphyrin synthase is present in the particulate fraction of the extracts of *C. tetanomorphum* but requires a heat-stable factor present in the soluble fraction. This soluble factor can be replaced by GSH. 6. Cobalt porphyrin synthase in the clostridial extract is inhibited by iodoacetamide and to a smaller extent by *p*-chloromercuribenzoate and *N*-ethylmaleimide. The haem synthases of *T. utilis* and *Micrococcus denitrificans* are also inhibited by various thiol reagents.

The structure of vitamin B_{12}, which was elucidated after the crystal-structure studies of Hodgkin *et al.* (1955) and the chemical studies of Bonnett *et al.* (1955), includes a corrin moiety (Fig. 1) that is the chelate of a porphyrin-like structure with a Co^{2+} ion. The corrin ring of vitamin B_{12}, like the porphyrin precursors of haem and chlorophyll pigments, can be synthesized enzymically from δ-aminolaevulic acid (Shemin, Corcoran, Rosenblum & Miller, 1956; Corcoran & Shemin, 1957; Bray & Shemin, 1963) or from porphobilinogen (Schwartz, Ikeda, Miller & Watson, 1959), suggesting a common pathway for the early reactions in the biosynthesis of cobalt, iron and magnesium tetrapyrroles. Although the insertion of Fe^{2+} ions into porphyrins by haem synthase (ferrochelatase) has been studied extensively (Yoneyama, Ohyama, Sugita & Yoshikawa, 1962; Labbe, Hubbard & Caughey, 1963; Porra & Jones, 1963*a*,*b*), little is known about the enzymic incorporation of Co^{2+} ions into vitamin B_{12}. Many workers have studied the ability of preparations from various haem-forming tissues and micro-organisms to insert not only Fe^{2+} but also Co^{2+} ions into porphyrins; the enzymic formation of cobalt porphyrins has been demonstrated with extracts of liver mitochondria (Labbe & Hubbard, 1961*a*,*b*; Yoneyama *et al.* 1962), avian erythrocyte preparations (Oyama, Sugita, Yoneyama & Yoshikaya, 1961; Johnson & Jones, 1964) and extracts from the micro-organisms *Thiobacillus X* and *Rhodopseudomonas spheroides* (Johnson & Jones, 1964).

To discover whether a specific enzyme is involved in the Co^{2+} ion-insertion reaction during the biosynthesis of vitamin B_{12} we have studied the enzymic formation of cobalt porphyrins by extracts of *Clostridium tetanomorphum*. This organism contains vitamin B_{12}, which is required for the energy-yielding fermentation of glutamate (Barker, Weissbach & Smyth, 1958), but it contains no detectable haems; like *Clostridium welchii*, therefore, *C. tetanomorphum* would also be expected to contain no haem-synthase activity (Porra & Jones, 1963*b*). The cobalt porphyrin-synthase

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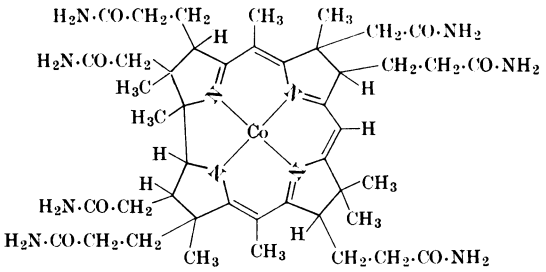

Fig. 1. Structure of the corrin moiety of vitamin B_{12}.

activity of *C. tetanomorphum* has been compared with the haem synthase of a yeast, *Torulopsis utilis*; yeasts are rich in haems when grown in aerobic conditions but contain little or no vitamin B_{12} (Ford & Hutner, 1955).

EXPERIMENTAL

Chemicals. Mesoporphyrin IX was supplied by Fluka A.-G., Basel, Switzerland. Coproporphyrin III, isolated from the culture fluids of *Rhodopseudomonas spheroides* (Lascelles, 1956), was a gift from Dr June Lascelles. Uroporphyrin III and the dimethyl esters of mesoporphyrin IX and deuteroporphyrin IX were gifts from Professor C. Rimington, University College Hospital Medical School, London. Deuteroporphyrin IX was prepared from the ester by hydrolysis in 6 N-HCl (Falk, 1964). Protoporphyrin IX was prepared from a crystalline sample of protohaemin IX by the method of Morrell & Stewart (1956), converted into the dimethyl ester by treatment with methanol containing 5% (v/v) of conc. HCl (Falk, 1964) and the ester then purified on a grade IV magnesium oxide column with chloroform–methanol solvents (Falk, 1961). Protoporphyrin IX was then obtained by hydrolysis of the purified ester in 6 N-HCl for 5 hr. at room temperature. Tween 80 (polyoxyethylene sorbitan mono-oleate) was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. All metal salts were AnalaR-grade reagents supplied by British Drug Houses Ltd., Poole, Dorset.

Organisms. The following organisms were used: *Clostridium tetanomorphum* strain 2909 (from the National Collection of Type Cultures); *Torulopsis utilis* provided by Dr F. Moss, University of New South Wales, Sydney, Australia; *Micrococcus denitrificans* supplied by Dr W. Verhoeven. Stock cultures of *C. tetanomorphum* were maintained in the glutamate medium of Barker *et al.* (1960); *T. utilis* was maintained on slopes of malt–yeast–agar; *M. denitrificans* was maintained as described by Chang & Morris (1962).

Growth of organisms. *C. tetanomorphum* was grown anaerobically at 37° for 20 hr. in the glutamate medium of Barker *et al.* (1960). *T. utilis* was grown aerobically at 30° for 18 hr. in the medium described by Ephrussi & Slonimski (1950), but the concentration of Difco yeast extract was lowered from 1% (w/v) to 0·5%. *M. denitrificans* was grown aerobically at 30° for 18 hr. in the succinate medium of Chang & Morris (1962).

Preparation of cell-free extracts. (*a*) Crude extracts. After being harvested and washed in 0·02 M-potassium phosphate buffer, pH 7·5, the organisms were suspended in fresh buffer to give a final concentration of approx. 150 mg. dry wt./ml. and then disrupted in the following manner: *C. tetanomorphum* and *M. denitrificans* were subjected to ultrasonic vibration for 8 and 5 min. respectively at 600 w and 25 kcyc./sec. with a Mullard ultrasonic generator type E7590B; the transducer assembly was cooled in an ice bath. *T. utilis* cells were broken by passing the cell suspension three times through the pressure cell described by Milner, Lawrence & French (1950) at 12000 lb./in.^{2}. Whole organisms and debris were removed from broken-cell suspensions by centrifugation at 6000 *g* for 15 min. at 4°. The supernatants were then dialysed overnight against two changes of 20 vol. of 0·02 M-potassium phosphate buffer, pH 7·5. These dialysed preparations, referred to below as crude extracts, contained 20–30 mg. of protein/ml.

(*b*) Soluble and particulate fractions. Crude extracts were fractionated into soluble and particulate preparations by centrifugation at 105000 *g* for 3 hr. at 4° in a Spinco model ultracentrifuge. The supernatant (soluble fraction) was removed by Pasteur pipette and the pellet resuspended in 0·02 M-potassium phosphate buffer, pH 7·5, and made up to a final volume equal to that of the supernatant fraction.

Assay of enzymes. (*a*) Haem synthase. This enzyme was assayed *in vacuo* in Thunberg tubes and haem determined from the difference spectrum of the reduced and oxidized pyridine haemochromogens (i.e. bispyridine ferro- and ferri-porphyrins), as described by Porra & Jones, 1963*a*,*b*). Two modifications were made: potassium phosphate buffer was replaced by tris buffer, pH 7·8, and GSH was omitted unless otherwise stated.

(*b*) Cobalt porphyrin synthase. This enzyme was assayed *in vacuo* in Thunberg tubes by the method of Johnson & Jones (1964). The method is similar to that used to measure haem synthase except for the substitution of $CoCl_2$ for $FeSO_4$. As in the haem-synthase assay, phosphate buffer was replaced by tris buffer, pH 7·8, and GSH was omitted unless otherwise stated. Cobalt porphyrin, like haem, is determined by measuring the difference spectrum of the bispyridine cobaltous and cobaltic porphyrins. For cobalt mesoporphyrin this spectrum revealed peaks at 389, 506 and 546·5mμ and troughs at 416 and 527mμ; the concentration was calculated by using a millimolar difference extinction coefficient ($\epsilon_{mM}^{546\cdot5} - \epsilon_{mM}^{527}$) value of 7·96, obtained by Johnson & Jones (1964) for cobalt deuteroporphyrin.

(*c*) Enzymic formation of other metalloporphyrins. During the studies of the metal ion specificity of the crude extracts of *C. tetanomorphum* and *T. utilis*, Mn^{2+}, Zn^{2+}, Cu^{2+} and Mg^{2+} ions were tested. Manganese porphyrin formation was assayed by the same technique as for haems and cobalt porphyrins, and the formation of zinc, copper and magnesium porphyrins was assayed *in vacuo* in Thunberg cuvettes by following the disappearance of porphyrin substrates (Johnson & Jones, 1964). The latter method was also used to measure the formation of cobalt uroporphyrin III because it proved difficult to form the bispyridine complex of this metalloporphyrin. The spectrum of the reaction mixture was recorded from 460 to 640 mμ on an Optica CF4DR recording spectrophotometer at intervals during the incubation. The utilization of porphyrin substrate was measured by following the disappearance of absorption bands I and IV and the formation of the metalloporphyrin as shown by the appearance of bands at about 540 and 580 mμ.

Examination of the haem content of crude extracts. Crude extracts of *C. tetanomorphum* were examined for the presence of haem before dialysis and the removal of whole cells and debris. To such crude extracts (1·0 ml.) was added 0·2 ml. of pyridine and 0·1 ml. of N-NaOH, and the solution obtained was divided equally between two microcuvettes (10 mm. light-path). Sodium dithionite was added to one cuvette and potassium ferricyanide to the other, and the difference spectrum recorded from 350 to 600 mμ. No peaks or troughs corresponding to the absorption bands of oxidized or reduced haemochromogens were visible.

Protein determinations. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

RESULTS

Properties of cobalt porphyrin synthase in C. tetanomorphum *extracts.* Mesoporphyrin IX, a porphyrin that does not normally occur in living tissues, was used as substrate in many of the following experiments on cobalt porphyrin formation, since it is a stable and soluble porphyrin and also because it is more readily converted into cobalt porphyrin by extracts of *C. tetanomorphum* than the other porphyrins that were tested (Table 1). The formation of cobalt mesoporphyrin increased linearly with time for at least 2 hr. and also increased linearly with concentration of the extract up to 60 mg. of protein. No cobalt porphyrin-synthase activity was observed in the presence of boiled extracts. The absorption spectrum of the pyridine complex of the product closely resembles that of the pyridine complex of cobalt mesoporphyrin (Whitten, Baker & Corwin, 1963); the Soret peaks of the cobaltous and cobaltic derivatives, as shown by the difference spectrum of the reduced and oxidized forms, were at 389 and 416 mμ respectively. The Soret peaks of the pyridine complex of a corrin derivative would be expected to be displaced some 40 or 50 mμ towards shorter wavelengths (Hill, Pratt & Williams, 1962; Bonnett, 1963).

Effect of pH *on the formation of cobalt mesoporphyrin by extracts of* C. tetanomorphum. Since porphyrins with carboxylic acid groups are amphoteric and their solubilities greatly influenced by pH, all experiments on the effect of pH were performed in the presence of a final concentration of 0·1% Tween 80 to ensure the complete solubility of the porphyrin substrate at all pH values tested (Porra & Jones, 1963*b*). This concentration of detergent increased the rate of cobalt mesoporphyrin formation by approx. 30% after a slight lag period, presumably by increasing the concentration of porphyrin substrate in solution. The non-enzymic formation of cobalt porphyrin in the presence of the detergent was low, about 4·4 mμmoles/hr. at pH 7·2 and 8·9. The pH–activity curve (Fig. 2) reveals an optimum pH region for cobalt porphyrin-synthase activity at pH 7·6–7·8.

Substrate specificities of crude extracts from C. tetanomorphum *and* T. utilis. To test whether *C. tetanomorphum* contains a cobalt porphyrin synthase but no haem synthase (see the introduction), the ability of crude extracts to insert Co^{2+} and Fe^{2+} ions into meso-, deutero- and protoporphyrins was determined (Table 1). The insertion of both Co^{2+} and Fe^{2+} ions was detected, but the rate of cobalt porphyrin formation far exceeded that of haem formation. Most of the extracts tested were unable to form detectable amounts of mesohaem. This suggests that crude extracts of *C. tetanomorphum* contain little or no haem synthase, but it is possible that cobalt porphyrin synthase can also use Fe^{2+} ions to a small extent. Crude extracts would not insert Co^{2+} ions into the dimethyl esters of deuteroporphyrin IX or of mesoporphyrin IX. Unlike the results obtained with the dicarboxylic porphyrin, mesoporphyrin IX (see above), the rate of non-enzymic incorporation of Co^{2+} ions into coproporphyrin III (tetracarboxylic) and uroporphyrin III (octacarboxylic) was high; no increase in the rate of cobalt copro-

Table 1. *Substrate specificity of the metalloporphyrin-forming enzymes of* C. tetanomorphum

Crude extract (1·0 ml.) was incubated for 2 hr. at 37° *in vacuo* with 200 mμmoles of porphyrin substrate, 400 mμmoles of $CoCl_2$ or $FeSO_4$ and 0·1% of Tween 80, as described in Fig. 2. The results are given as specific activities.

	Metalloporphyrin formed (mμmoles/hr./mg. of protein)	
Porphyrin substrate	Cobalt porphyrin	Haem
Mesoporphyrin IX	3·0	0·1
Deuteroporphyrin IX	1·7	0·4
Protoporphyrin IX	1·2	0·0

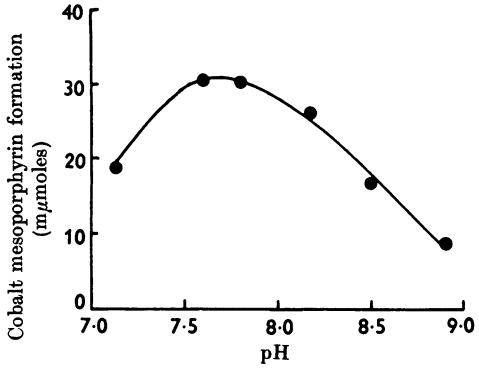

Fig. 2. Effect of pH on cobalt mesoporphyrin formation by extracts of *C. tetanomorphum*. Crude extract (1·0 ml.) was incubated at 37° *in vacuo* in Thunberg tubes with 200 mμmoles of mesoporphyrin, 400 mμmoles of $CoCl_2$, 400 mμmoles of tris and 0·1% of Tween 80. The final volume was 4·5 ml. Incubations of 1½ hr. duration were started by tipping the metal salt from the side arm. Cobalt mesoporphyrin formation was measured as described in the Experimental section.

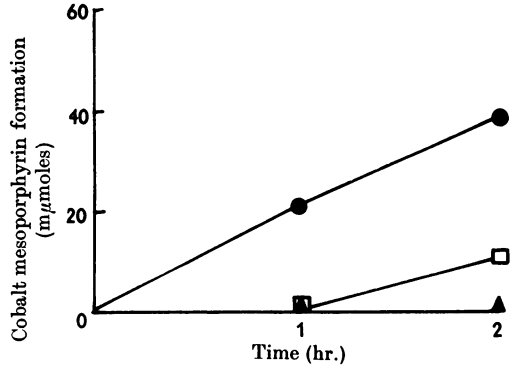

Fig. 3. Effect of oxygen on cobalt porphyrin-synthase activity in extracts of *C. tetanomorphum*. Crude extract (1·0 ml.) was incubated at pH 7·8 with mesoporphyrin, $CoCl_2$ and Tween 80 at 37°, as described in Fig. 2. Cobalt mesoporphyrin formation *in vacuo* (●), aerobically in stationary tubes (□) and aerobically in tubes shaken at 120 oscillations/min. with an amplitude of 5 cm. (▲) was determined as described in the Experimental section.

Table 2. *Substrate specificity of the metalloporphyrin-forming enzymes of* T. utilis

Crude extract (0·5 ml.) was incubated for 1 hr. at 30° *in vacuo* with 200 mμmoles of porphyrin substrate, 400 mμmoles of $FeSO_4$ or $CoCl_2$ and 0·1% of Tween 80, as described in Fig. 2. The results are given as specific activities.

Porphyrin substrate	Metalloporphyrin formed (mμmoles/hr./mg. of protein)	
	Cobalt porphyrin	Haem
Deuteroporphyrin IX	9·0	5·8
Mesoporphyrin IX	6·8	3·4
Protoporphyrin IX	0·8	0·2

porphyrin or cobalt uroporphyrin formation was observed in the presence of enzyme extract. These crude extracts were also unable to catalyse the insertion of Mn^{2+}, Mg^{2+}, Zn^{2+} or Cu^{2+} ions into mesoporphyrin IX.

Crude extracts of *T. utilis*, as expected, could form haems more readily than could extracts from *C. tetanomorphum*, but their ability to form cobalt porphyrins was also high (Table 2). The insertion of Co^{2+} ions by *T. utilis* extracts, like that of Fe^{2+} ions, occurs more readily with deuteroporphyrin IX than with mesoporphyrin IX (Table 2), but the insertion of Co^{2+} ions by extracts of *C. tetanomorphum* follows the reverse pattern (Table 1).

Effect of oxygen on cobalt porphyrin-synthase activity in extracts of C. tetanomorphum. A requirement for strictly anaerobic conditions for the insertion of Fe^{2+} ions into porphyrins by soluble extracts of liver mitochondria was found by Porra & Jones (1963*a*). However, evidence has been found (A. Johnson & O. T. G. Jones, personal communication) that the insertion of metal ions other than Fe^{2+} by extracts of *R. spheroides*, *Thiobacillus X* and chicken erythrocytes is not inhibited by oxygen. The inhibitory effect of oxygen on cobalt porphyrin-synthase activity in extracts of *C. tetanomorphum* is not as severe as reported for haem synthase of liver mitochondria. If the incubation tubes were not shaken, cobalt porphyrin formation was observed under aerobic conditions after a short lag phase. Nevertheless, the results in Fig. 3 show that shaking the tubes to ensure more vigorous aeration completely inhibited cobalt porphyrin-synthase activity.

Particulate nature of cobalt porphyrin synthase and the effect of thiol reagents. It has been shown by Lochhead & Goldberg (1961) that haem synthase in mammalian tissues is located mainly in the mitochondria. Our investigation of the soluble and particulate fractions of *T. utilis* revealed that over 80% of the haem-synthase activity of this organism was, likewise, associated with particulate matter. A similar study of cobalt porphyrin synthase in *C. tetanomorphum* produced unexpected results (Table 3): neither the soluble nor the particulate fraction alone exhibited any ability to catalyse cobalt porphyrin formation, but on mixing the two fractions activity was observed. Activity was partially restored if boiled soluble extract was added to the particulate fraction; the addition of

GSH could replace the requirement for the supernatant fraction (Table 3).

Labbe & Hubbard (1960) showed that haem synthase in mammalian tissues is inhibited by thiol reagents. Such reagents also inhibit the catalysis of mesohaem formation by crude extracts of *T. utilis* and *M. denitrificans* (Table 4); the latter organism is rich in haem-synthase activity (Porra & Lascelles, 1965). However, both Neuberger & Tait (1964) and Johnson & Jones (1964) have reported that the enzymic insertion of other metal ions into porphyrins is less sensitive to thiol reagents. Since GSH restored cobalt porphyrin-synthase activity to particulate fractions of *C. tetanomorphum*, and since thiol reagents inhibit the formation of cobalt mesoporphyrin by crude extracts of this organism (Table 5), cobalt porphyrin synthase does appear to be a thiol enzyme.

Table 3. *Particulate nature of cobalt porphyrin synthase of* C. tetanomorphum *and the effect of glutathione*

Particulate and soluble fractions (1·0 ml.) were incubated for 1½ hr. *in vacuo* at 37° in the presence of mesoporphyrin, $CoCl_2$ and Tween 80, as described in Fig. 2. Boiled fractions had been heated at 100° for 8 min.

Preparation used	Additions	Cobalt mesoporphyrin formed (mμmoles)
Supernatant	—	0
Particles	—	0
Particles	Supernatant	30·8
Particles	Boiled supernatant	18·2
Particles	GSH (20 μmoles)	34·5
Supernatant	GSH (20 μmoles)	0

DISCUSSION

Although information is available on the biosynthesis of various parts of the vitamin B_{12} molecule (for reviews see White, 1962; Brown & Reynolds, 1963; Bernhauer, Müller & Wagner, 1964), it is not known at what stage the Co^{2+} ion is incorporated into the corrin ring. The present work suggests that there is a specific enzyme, cobalt porphyrin synthase, in crude extracts of *C. tetanomorphum* that catalyses the incorporation of Co^{2+} ions into tetrapyrrole pigments during the biosynthesis of vitamin B_{12}. Like *C. welchii* (Porra & Jones, 1963*b*), *C. tetanomorphum* extracts contain little or no haem-synthase activity to obscure the results obtained during the investigation of the properties of cobalt porphyrin synthase. Cobalt porphyrin synthase was able to insert Co^{2+} ions into several dicarboxylic porphyrins, but

Table 4. *Effect of thiol reagents on haem-synthase activity of* T. utilis *and* M. denitrificans

Crude extracts of *T. utilis* (0·5 ml.) and *M. denitrificans* (1·0 ml.) were incubated for 60 min. and 30 min. respectively *in vacuo* at 30° with mesoporphyrin, $FeSO_4$ and Tween 80, as described in Table 1. Before the $FeSO_4$ was tipped in from the side arms the rest of the reaction mixture was incubated for 10 min. at 30° with inhibitor.

Source of crude extract	Inhibitor	Final concn. of inhibitor (mM)	Mesohaem formed (mμmoles)	Percentage inhibition
T. utilis	None	—	56·5	—
	Iodoacetamide	0·45	53·5	5·3
	p-Chloromercuribenzoate	0·125	53·5	5·3
	N-Ethylmaleimide	0·45	35·5	31·9
M. denitrificans	None	—	89·0	—
	N-Ethylmaleimide	0·45	37·0	58·5
	p-Chloromercuribenzoate	0·125	6·5	92·5

Table 5. *Effect of thiol reagents on cobalt porphyrin formation by extract of* C. tetanomorphum

Crude extract (1·0 ml.) was incubated for 1½ hr. at 37° with mesoporphyrin, $CoCl_2$ and Tween 80, as described in Fig. 2. Before the $CoCl_2$ was tipped in from the side arm the rest of the reaction mixture was incubated for 10 min. at 37° with inhibitor.

Inhibitor	Final concn. of inhibitor (mM)	Cobalt mesoporphyrin formed (mμmoles)	Percentage inhibition
None	—	77	—
N-Ethylmaleimide	0·45	72	6·0
p-Chloromercuribenzoate	0·125	69	10·5
Iodoacetamide	0·45	46	40·5

these porphyrins became inactive as substrates when the carboxylic acid groups were masked by esterification. The enzyme was also unable to use coproporphyrin III or uroporphyrin III. The inability to use the latter substrate is a little surprising. Of the known naturally occurring tetrapyrroles uroporphyrin III resembles the corrin ring of vitamin B_{12} most closely, and it is conceivable that the β-substituents of the corrin ring, with the exception of the methyl groups derived from methionine (Bray & Shemin, 1963), are all derived from the acetic acid and propionic acid groups of uroporphyrin III.

It is possible that the natural substrate of cobalt porphyrin synthase is not a porphyrin but a linear tetrapyrrole formed during the conversion of porphobilinogen into the corrin ring, and that ring closure follows the insertion of the Co^{2+} ion. This would be analogous to the first chemical synthesis of the corrin ring described by Eschenmoser (1965), in which the direct linkage between rings I and IV is achieved first, followed by the insertion of a Co^{2+} ion and then by the formation of the final bridge between rings I and II. Johnson (1965), however, has demonstrated that Co^{2+} ions can also be inserted into a closed corrin-like ring.

Crude extracts of *T. utilis* readily formed both haems and cobalt porphyrins (Table 2). This suggests the following two alternatives: either that extracts of *T. utilis* contain two separate enzymes, haem synthase and cobalt porphyrin synthase, or that the extracts contain the one enzyme, haem synthase, which exhibits little discrimination between Fe^{2+} and Co^{2+} ions. In support of the latter alternative are the observations that chicken erythrocyte preparations (Johnson & Jones, 1964) and mammalian liver preparations (Labbe & Hubbard, 1961*a*,*b*; Yoneyama *et al.* 1962) readily form both haems and cobalt porphyrins; but, though both tissues are able to form haems *in vivo*, neither is able to synthesize vitamin B_{12}.

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